Association of Effector *Six6* with Vascular Wilt Symptoms Caused by *Fusarium oxysporum* on Soybean

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Accepted for publication 24 May 2016.

ABSTRACT

Lanubile, A., Ellis, M. L., Marocco, A., and Munkvold, G. P. 2016. Association of effector *Six6* with vascular wilt symptoms caused by *Fusarium oxysporum* on soybean. *Phytopathology* 106:1404-1412.

The *Fusarium oxysporum* species complex (FOSC) is a widely distributed group of fungi that includes both pathogenic and nonpathogenic isolates. In a previous study, isolates within the FOSC collected primarily from soybean were assessed for the presence of 12 fungal effector genes. Although none of the assayed genes was significantly associated with wilt symptoms on soybean, the secreted in xylem 6 (*Six6*) gene was present only in three isolates, which all produced high levels of vascular wilt on soybean. In the current study, a collection of *F. oxysporum* isolates from soybean

roots and *F. oxysporum* f. sp. *phaseoli* isolates from common bean was screened for the presence of the *Six6* gene. Interestingly, all isolates for which the *Six6* amplicon was generated caused wilt symptoms on soybean, and two-thirds of the isolates showed high levels of aggressiveness, indicating a positive association between the presence of the effector gene *Six6* and induction of wilt symptoms. The expression profile of the *Six6* gene analyzed by quantitative reverse-transcription polymerase chain reaction revealed an enhanced expression for the isolates that caused more severe wilt symptoms on soybean, as established by the greenhouse assay. These findings suggest the suitability of the *Six6* gene as a possible locus for pathogenicity-based molecular diagnostics across the various formae speciales.

Fusarium oxysporum Schltdl. is a soilborne fungal species present worldwide that includes both plant-pathogenic and nonpathogenic strains. The pathogenic strains are devastating plant pathogens causing wilt and root rot diseases across a wide array of hosts, including economically important crops such as cotton, tomato, banana, and several legumes such as soybean and common bean (Michielse and Rep 2009). Phytopathogenic strains of *F. oxysporum* have been subdivided into over 100 different host-specific forms (formae speciales), making the *F. oxysporum* species complex (FOSC) a very appealing system to study the relationships between pathogenicity and host specificity (Baayen et al. 2000; Di Pietro et al. 2003; Lievens et al. 2008).

F. oxysporum is one of the most prevalent *Fusarium* spp. associated with soybean root rot and seedling diseases in Iowa and other soybean-producing regions in North America (Díaz Arias et al. 2013a,b; Killebrew et al. 1993; Rizvi and Yang 1996). In a recent work, the phenotypic and genotypic diversity was explored within the FOSC collected from soybean roots, based on phylogenetic analyses of conserved genes such as the translation elongation factor 1- α (*tef1 α*) gene and the mitochondrial small subunit (mtSSU) (Ellis et al. 2014). The results indicated that the tested isolates were polyphyletic in origin, belonging to five clades within the FOSC, including *F. commune*, previously within the FOSC (Skovgaard et al. 2001). Isolate aggressiveness varied within and among clades, with only a weak association between clade and low aggressiveness for one clade (Ellis et al. 2014).

F. oxysporum f. sp. *phaseoli* infects some species of the genus *Phaseolus*, mainly *Phaseolus vulgaris* and *P. coccineus* (de Vega-Bartol

et al. 2011), and is one of the principal pathogens limiting dry bean production worldwide. The *Fusarium* transcription factor (*fft1*) has been identified as being associated with highly virulent *F. oxysporum* f. sp. *phaseoli* strains toward common bean (de Sousa et al. 2015; Ramos et al. 2007). A rapid and accurate real-time polymerase chain reaction (PCR) assay has been developed to detect highly virulent *F. oxysporum* f. sp. *phaseoli* isolates from common bean seed using primers based on the *fft1* (de Sousa et al. 2015).

The identification of pathogenic *F. oxysporum* isolates is traditionally based on pathogenicity testing, which is time consuming and laborious. Ideally, it would be possible to distinguish formae speciales based on DNA sequences directly related to host-specific pathogenicity or nonpathogenicity (Lievens et al. 2008). Unfortunately, molecular discrimination among *F. oxysporum* isolates is seriously complicated by the polyphyletic nature within the FOSC on different hosts, including soybean (Gordon and Martyn 1997; Lievens et al. 2008).

Effectors are typically pathogen proteins and small molecules that promote host colonization (virulence factors or toxins) or modulate plant immunity (avirulence factors and elicitors) or both (Dodds and Rathjen 2010; Hogenhout et al. 2009). Fungal effector genes fall into several classes, including genes coding infection structures, cuticle- and cell-wall-degrading enzymes, fungal toxins, signal cascade components, and suppression of plant immunity (Hogenhout et al. 2009; Houterman et al. 2007; Idnurm and Howlett 2001; Lanubile et al. 2015). Additional fungal effector genes were identified from xylem sap of infected plants, encompassing the “secreted in xylem” (SIX) proteins. Currently, 14 *Six* genes have been identified in *F. oxysporum* f. sp. *lycopersici*, some of which have been successfully used to differentiate between three races of *F. oxysporum* f. sp. *lycopersici* (Houterman et al. 2008; Lievens et al. 2009; Rep et al. 2004; Schmidt et al. 2013). The biological function of SIX proteins remains largely unknown and their amino acid sequences display little or no homology with other known proteins. However, for SIX1, SIX3, and SIX4 proteins in *F. oxysporum* f. sp. *lycopersici*, interactions with host resistance (*R*) genes have been

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*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures are published online.

<http://dx.doi.org/10.1094/PHYTO-03-16-0118-R>
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demonstrated in tomato. Recently, *Six6* was shown to contribute to pathogenicity of *F. oxysporum* f. sp. *lycopersici* and it specifically suppresses I-2-mediated cell death upon transient expression in *Nicotiana benthamiana* (Gawehns et al. 2014). Most of the *Six* genes are localized on chromosome 14 of *F. oxysporum* f. sp. *lycopersici*, including *Six6*, and, consequently, it is referred to as a “pathogenicity” chromosome (Ma et al. 2010; Schmidt et al. 2013).

The *Six* genes have been detected in formae speciales other than *F. oxysporum* f. sp. *lycopersici*. Lievens et al. (2009) showed that the *Six6* and *Six7* effectors, identified originally in isolates collected from tomato, were present in many other formae speciales, suggesting that they may play a more general role in pathogenicity. The work from Chakrabarti et al. (2011) suggests that the *Six6* effector might be used as a diagnostic marker for populations of *F. oxysporum* from cotton in Australia. They also suggested that a homolog of the *fif1* transcription factor, identified from common bean isolates, might be linked with the *Six6* effector from the cotton isolates. However, this marker was not useful for populations of *F. oxysporum* in other countries.

In our previous study, 80 isolates collected primarily from soybean were assessed for the presence of 12 fungal effector genes: *Fmk1*, *Fow1*, *Pda1*, *PelA*, *PelD*, *Pep1*, *Prt1*, *Rho1*, *Sge1*, *Six1*, *Six6*, and *Snf1* (Ellis et al. 2016). Fungal effector genes *Fmk1*, *Fow1*, *PelA*, *Rho1*, *Sge1*, and *Snf1* were present in all isolates screened, while *Pda1*, *PelD*, *Pep1*, *Prt1*, *Six1*, and *Six6* were dispersed among isolates. Of these, *Fmk1*, *Fow1*, *Pda1*, *PelA*, *Rho1*, *Sge1*, and *Snf1* corresponded to previously designated clades based on *tef1α* and mtSSU sequences (Ellis et al. 2014, 2016). The same isolates were also tested in the greenhouse for their ability to cause wilt symptoms on soybean. From the analysis of molecular variance, there was no significant association between any of the assayed genes and wilt symptoms (Ellis et al. 2016). However, the *Six6* gene was only present in three previously known wilt isolates from soybean, common bean, and tomato, all of which produced high levels of vascular wilt on soybean in our study.

The objectives of this study were to (i) determine the phylogenetic relationship between *F. oxysporum* isolates from soybean roots and *F. oxysporum* f. sp. *phaseoli* isolates from common bean on the basis of the *tef1α* and mtSSU loci, (ii) screen isolates for the presence of the fungal effector *Six6*, and (iii) assess the association between isolate aggressiveness (vascular wilt symptom severity) and the presence and expression level of the *Six6* gene.

MATERIALS AND METHODS

Fungal isolates. In all, 22 isolates of *F. oxysporum* f. sp. *phaseoli* collected from common bean, 15 isolates of *F. oxysporum*, and 5 isolates of *F. commune* collected from soybean were used in this study and are listed in Table 1 with their geographical origin. One isolate of *F. oxysporum* f. sp. *lycopersici* (FGSC 9935, NRRL34936) collected from tomato, one isolate of *F. oxysporum* f. sp. *tracheiphilum* (ATCC16608) collected from cowpea, one isolate of *F. oxysporum* f. sp. *vasinfectum* (CML1098) collected from cotton, one isolate of *F. oxysporum* from coffee (CML755), and one isolate of *F. oxysporum* (FGSC 10445, NRRL54002) that was characterized as a biocontrol were also used in the study and listed in Table 1. The isolates collected from common bean were previously tested for their pathogenicity toward common bean, as described by de Sousa et al. (2015), whereas the isolates collected from soybean were selected based on their ability to produce vascular wilt symptoms on soybean from a previous study by Ellis et al. (2016) (Table 1). Isolates were maintained on silica in the dark at 5°C at the Iowa State University (ISU) Seed Science Center in Ames.

DNA extraction and sequencing. The DNA for each isolate was prepared following the method previously described by Ellis et al. (2014). Briefly, isolates were grown on potato dextrose agar (PDA) for 5 days at 25°C with a 12-h photoperiod. A sterile loop was used to scrape and transfer mycelium and conidia of each isolate to a

250-ml glass flask containing 50 ml of liquid growth media (3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 20 g of dextrose [O'Donnell 1992], plus 2 g of NH₄SO₄ in 1 liter of water). The inoculated medium was placed on an Innova 2100 platform shaker (New Brunswick Scientific Co., Inc., Enfield, CT) at 100 rpm and incubated at ambient temperature for 3 to 5 days. Sterile Miracloth (EMD Biosciences Inc., La Jolla, CA) was used to harvest the mycelium, which was then frozen, lyophilized, and ground in a porcelain mortar and pestle (CoorsTek, Inc., Golden, CO). The fungal DNA was extracted as described by Zelaya-Molina et al. (2011). DNA was evaluated by measuring the concentration and the ratios of absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀) and 260 and 230 nm (A₂₆₀/A₂₃₀) with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and diluted to 10 ng of genomic DNA using nuclease-free water (Ambion, Austin, TX).

Sequences for *tef1α* and mtSSU for the 15 isolates collected from soybean were previously obtained in the study by Ellis et al. (2014). The *tef1α* gene and mtSSU for the remaining 27 isolates from other hosts were amplified using a 25-μl PCR mix containing 20 ng of genomic DNA, 5 μl of 5× Colorless GoTaq Reaction buffer (Promega Corp., Madison, WI), 2.5 μl of 25 mM MgCl₂ (Promega Corp.), 1.5 μl of 1.3 mM each dNTP (Fermentas/Thermo Fisher Scientific, Inc., Pittsburgh), 0.5 μl of GoTaq Taq polymerase (Promega Corp.), 2.5 μl each of a 5 pmol concentration of primers, and 9.5 μl of sterile water. PCR were carried out using a T100 Thermal Cycler (Bio-Rad, Hercules, CA). For amplification of *tef1α* and mtSSU, the following primers were used for each gene, respectively: EF-1H (ATGGGTAAGGAAGACAAGAC) and EF-2T (GGAAGTACCAGTGATCATGTT) (O'Donnell et al. 1998), and MS1 (CAGCAGTCAAGAATATTGCAATG) and MS2 (GCGGATTATCGAATTAAATAAC) (White et al. 1990). The PCR parameters for the *tef1α* gene were 94°C for 1 min; followed by 35 cycles of a denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. For the mtSSU, the PCR parameters were 97°C for 1 min; followed by 35 cycles of a denaturation at 94°C for 35 s, annealing at 52°C for 55 s, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min (Laurence et al. 2012). All 47 isolates were screened for the presence of the *Six6* gene using the same PCR parameters described above. The PCR conditions consisted of an initial denaturing at 94°C for 2 min; followed by 32 cycles of 94°C for 45 s, annealing at 61°C for 45 s, and 72°C for 45 s; and a final extension step of 72°C for 5 min (Covey et al. 2014). The primers for amplification of the *Six6* gene were *Six6F* (AGAAGAGTTTGAACCACCGAAGGCG) and *Six6R* (ACTGGAAATTGTCGCTGTGAAGTACG) (Covey et al. 2014). Product integrity of the PCR product was analyzed using gel electrophoresis on a 2% agarose containing 1% DNA Safe (Applied Biological Materials, Inc., Richmond, BC, Canada). The bands were visualized using the Bio-Rad Gel Doc System and sized using the TriDye 100-bp ladder (New England BioLabs Inc., Ipswich, MA). The PCR product was purified using Exosap-IT (USB Corp., Cleveland), following the manufacturer's protocol.

The purified PCR amplicons were sequenced at the DNA Facility of the ISU Office of Biotechnology in Ames using the ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The raw sequence data for each of the genes was aligned and edited using CodonCode Aligner 3.7.1 (CodonCode Corp, Dedham, MA). The sequences for *Six6* gene were compared with known DNA sequences generated by the study by Covey et al. (2014) that were obtained from the National Center for Biotechnology Information (NCBI) database (<https://ncbi.nlm.nih.gov>).

Phylogenetic analysis. Phylogenies were inferred for *tef1α*, mtSSU, the combined dataset (sequence data for both the *tef1α* and mtSSU genes), and the effector gene *Six6*. For the final analyses of *tef1α* and mtSSU, 54 nucleotide sequences for each gene were used, including representative sequences from six clades within the FOSC, including *F. commune*, collected from NCBI GenBank from

studies by O'Donnell et al. (2004) and Ellis et al. (2014). For the analysis of the *Six6* gene, 22 nucleotide sequences were used, including sequences obtained from NCBI GenBank, as reported by Chakrabarti et al. (2011), Covey et al. (2014), and Lievens et al. (2009). Gene sequences obtained from this study were submitted to GenBank with the following accessions: KP681649 to KP681661 for *Six6*, KP693820 to KP693863 for *mtSSU*, and KP693864 to KP693907 for *tef1α*. Sequences were aligned using ClustalW

(Thompson et al. 1994). Phylogenetic analyses were performed using the maximum parsimony and maximum-likelihood methods in MEGA 6 (Tamura et al. 2013) and Bayesian inference using the MrBayes plugin of Geneious (Huelsenbeck and Ronquist 2001). For the maximum parsimony analyses, heuristic searches for the most parsimonious trees were conducted with 10 random addition replicates and tree-bisection-reconstruction (Nei and Kumar 2000) with search level 1. The best-fit model of nucleotide substitution for

TABLE 1. Characteristics of isolates in the *Fusarium oxysporum* species complex evaluated in this study

Species	Isolate	Host	Origin ^a	Clade	<i>tef1α</i> ^b	<i>Six6</i>	<i>ftf1</i> ^c	<i>N</i> ^d	Score ^e	Wt (g) ^f	Stem ^g	Root ^g	Agrv ^h
<i>F. oxysporum</i>													
f. sp. <i>phaseoli</i>	9455	<i>Phaseolus vulgaris</i>	Brazil	3	3B	+	+	3	1.5	5.98	0	4	L
f. sp. <i>phaseoli</i>	9840	<i>P. vulgaris</i>	Brazil	2	2A	–	–	0	1	6.98	0	0	NP
f. sp. <i>phaseoli</i>	2556-1	<i>P. vulgaris</i>	Brazil	3	3E	–	+	0	1	6.67	0	0	NP
f. sp. <i>phaseoli</i>	ATCC 18131	<i>P. vulgaris</i>	California	3	3B	+	+	10	1.5–4 (2.45 ± 0.24)	2.84	10	10	H
f. sp. <i>phaseoli</i>	CML144	<i>P. vulgaris</i>	Brazil	2	2A	–	–	3	1.5–2 (1.66 ± 0.14)	7.15	0	0	NP
f. sp. <i>phaseoli</i>	FOP16	<i>P. vulgaris</i>	Colorado	3	3B	+	+	3	1.5	5.92	0	5	M
f. sp. <i>phaseoli</i>	FOP31	<i>P. vulgaris</i>	Netherlands	3	3A	+	+	10	2–5 (3.60 ± 0.23)	2.49	10	10	H
f. sp. <i>phaseoli</i>	FOP48	<i>P. vulgaris</i>	Colorado	3	3C	–	–	0	1	7.38	0	0	NP
f. sp. <i>phaseoli</i>	FOP52	<i>P. vulgaris</i>	Colorado	3	3B	+	+	10	2–5 (4.50 ± 0.29)	2.01	10	10	H
f. sp. <i>phaseoli</i>	FOP58	<i>P. vulgaris</i>	Spain	2	2F	+	+	10	2–5 (3.80 ± 0.27)	2.64	10	10	H
f. sp. <i>phaseoli</i>	FOP8	<i>P. vulgaris</i>	Colorado	3	3B	+	+	10	2–5 (3.65 ± 0.43)	3.41	10	10	H
f. sp. <i>phaseoli</i>	FOPS018	<i>P. vulgaris</i>	Brazil	2	2A	–	+	0	1	7.46	0	0	NP
f. sp. <i>phaseoli</i>	FOPS019	<i>P. vulgaris</i>	Brazil	2	2A	+	+	1	2	6.56	0	5	M
f. sp. <i>phaseoli</i>	FOPS023	<i>P. vulgaris</i>	Brazil	2	2A	+	+	6	1.5–5 (2.08 ± 0.58)	5.94	1	2	L
f. sp. <i>phaseoli</i>	FOPS025	<i>P. vulgaris</i>	Brazil	2	2E	+	+	2	1.5	6.01	1	7	M
f. sp. <i>phaseoli</i>	FOPS026	<i>P. vulgaris</i>	Brazil	2	2E	+	+	0	1	6.29	3	6	M
f. sp. <i>phaseoli</i>	LAPS152	<i>P. vulgaris</i>	Brazil	2	2G	–	+	0	1	6.68	0	0	NP
f. sp. <i>phaseoli</i>	LAPS153	<i>P. vulgaris</i>	Brazil	3	3E	–	+	8	1.5–3 (1.68 ± 0.18)	6.05	0	0	NP
f. sp. <i>phaseoli</i>	LAPS154	<i>P. vulgaris</i>	Brazil	3	3E	–	+	3	1.5	6.80	0	0	NP
f. sp. <i>phaseoli</i>	LAPS155	<i>P. vulgaris</i>	Brazil	3	3E	–	+	3	1.5–2 (1.66 ± 0.14)	7.52	0	0	NP
f. sp. <i>phaseoli</i>	LAPS156	<i>P. vulgaris</i>	Brazil	3	3E	–	+	0	1	5.92	0	0	NP
f. sp. <i>phaseoli</i>	LAPS157	<i>P. vulgaris</i>	Brazil	3	3E	–	–	0	1	7.05	0	0	NP
f. sp. <i>lycopersici</i>	NRRL34936	<i>Solanum lycopersicum</i>	Illinois	3	3F	+	NA	0	1	6.37	2	4	M
f. sp. <i>tracheiphilum</i>	ATCC16608	<i>Vigna unguiculata</i>	Virginia	2	2D	–	NA	0	1	6.75	0	0	NP
f. sp. <i>vasinfectum</i>	CML1098	<i>Gossypium hirsutum</i>	Brazil	2	2A	–	–	2	1.5	5.85	0	0	NP
<i>F. oxysporum</i>	CML755	<i>Coffea arabica</i>	Brazil	2	2A	–	–	0	1.5	6.82	0	0	NP
<i>F. oxysporum</i>	NRRL54002	Soil (biocontrol)	France	3	3A	–	NA	0	1	6.07	0	0	NP
<i>F. oxysporum</i>	ATCC62913	<i>Glycine max</i>	Georgia	2	2C	+	NA	10	2–5 (3.50 ± 0.45)	2.27	10	10	H
<i>F. oxysporum</i>	FO132	<i>G. max</i>	Iowa	2	2A	–	NA	0	1	7.01	0	0	NP
<i>F. oxysporum</i>	FO23	<i>G. max</i>	Iowa	5	5A	–	–	1	1.5	6.91	0	0	NP
<i>F. oxysporum</i>	FO29	<i>G. max</i>	Iowa	3	3D	–	NA	10	1.5	5.89	1	1	L
<i>F. oxysporum</i>	FO3	<i>G. max</i>	Iowa	3	3A	–	–	1	1.5	6.89	0	3	M
<i>F. oxysporum</i>	FO35	<i>G. max</i>	Iowa	5	5B	–	NA	6	1.5	6.39	0	0	NP
<i>F. oxysporum</i>	FO43	<i>G. max</i>	Iowa	5	5A	–	NA	2	1.5	6.52	0	0	NP
<i>F. oxysporum</i>	FO5	<i>G. max</i>	Iowa	3	3A	–	NA	4	1.5	6.06	0	1	L
<i>F. oxysporum</i>	FO57	<i>G. max</i>	Iowa	5	5A	–	NA	5	1.5	7.14	1	2	L
<i>F. oxysporum</i>	FO59	<i>G. max</i>	Iowa	3	3D	–	NA	3	1.5	7.43	0	3	M
<i>F. oxysporum</i>	FO6	<i>G. max</i>	Iowa	2	2A	–	NA	6	1.5	5.80	1	3	M
<i>F. oxysporum</i>	FO62	<i>G. max</i>	Iowa	5	5A	–	NA	3	1.5–3 (1.68 ± 0.18)	5.56	1	3	M
<i>F. oxysporum</i>	FO63	<i>G. max</i>	Iowa	2	2A	–	–	2	1.5	6.31	0	1	L
<i>F. oxysporum</i>	FO79	<i>G. max</i>	Iowa	2	2B	–	NA	5	1.5	6.58	1	1	L
<i>F. oxysporum</i>	NE-5-18sm	<i>G. max</i>	Nebraska	3	3D	–	NA	8	1.5	6.51	0	3	M
<i>F. commune</i>	07-242	<i>G. max</i>	Minnesota	6	6A	–	NA	7	1.5	6.24	2	5	H
<i>F. commune</i>	FO61	<i>G. max</i>	Iowa	6	6A	–	NA	6	1.5	6.91	0	4	M
<i>F. commune</i>	FO72	<i>G. max</i>	Iowa	6	6A	–	NA	7	1.5	6.58	0	4	M
<i>F. commune</i>	FO93	<i>G. max</i>	Iowa	6	6B	–	NA	6	1.5–5 (1.68 ± 0.18)	4.46	6	9	H
<i>F. commune</i>	FO117	<i>G. max</i>	Iowa	6	6A	–	NA	5	1.5	7.02	5	8	H
...	Control ⁱ	...	...	...	...	...	...	0	1	8.34	0	0	...

^a Geographic origin.

^b Sequence designation for unique *tef1α* gene sequences; number indicates clade, followed by consecutive letter designations within clades.

^c Data from de Sousa et al. (2014) for the *Fusarium* transcription factor 1 gene (*ftf1*). NA = data were not collected for these isolates.

^d Number out of 10 soybean plants with foliar symptoms.

^e Disease score. For the isolates showing a range of disease scores, the average ± standard error (SE) is indicated after the range values.

^f Root weight: average of 10 soybean plants.

^g Stem and root lesions: number out of 10 soybean plants with vascular discoloration in the stem and root.

^h Aggressiveness toward soybean was categorized as nonpathogenic (NP) (no plants with symptoms), low (L) (symptom incidence ≥1% and <30%), moderate (M) (symptom incidence ≥30% and <70%), or high (H) (≥70% symptom incidence), based on stem and root lesion comparisons with known check isolates.

ⁱ Noninoculated control.

each gene and the combined dataset was determined in MEGA 6 for the maximum-likelihood and Bayesian analyses. For the maximum-likelihood analyses, the Kimura two-parameter model (Kimura 1980) plus the γ correction for among-site variation (K2+G) was used for the *tef1 α* and *Six6* genes, the Tamura three-parameter model (T92) (Tamura 1992) for the mtSSU, and the T92 model plus the γ correction for among-site variation (T92+G) was used for the combined dataset. For the maximum parsimony and maximum-likelihood analyses, gaps were treated as missing data and relative support for branches was estimated with 1,000 bootstrap replications (Felsenstein 1985). For the Bayesian analyses, the likelihood parameter settings (lset), number of substitution types (nst = 2), with a γ distribution (rates = γ), and the number of discrete categories used to approximate the γ distribution

(Ngammatat = 4) were selected for all genes and combined dataset. The Bayesian inference trees were constructed using three hot and one cold Markov chain Monte Carlo iterations with 1,100,000 generations, with sampling every 200 generations, and the first 110,000 generations were discarded as the chains converged.

Greenhouse Fusarium wilt assay. The 47 isolates were tested in the greenhouse for their ability to cause wilt symptoms in soybean, as previously described by Ellis et al. (2016). Greenhouse experiments were established at the ISU Department of Plant Pathology and Microbiology greenhouse facilities in Ames from September to November 2014. Briefly, two soybean seeds of susceptible ‘MN1805’ (Cruz et al. 2013) were planted in trays with 48 cells containing perlite (Midwest Perlite Co., Appleton, WI). The

Fig. 1. Maximum parsimony phylogenetic tree of *Fusarium oxysporum* and *F. commune* isolates from soybean and common bean inferred from the combined translation elongation factor gene and the mitochondrial small subunit sequence data. All sequences are followed by their GenBank accessions. Bootstrap values for the maximum parsimony and maximum-likelihood analyses (1,000 replications) and Bayesian posterior probabilities are indicated as percentages, in that order, at the internodes. *F. redolens* strain NRRL31075 was used to root the tree. Fop = *F. oxysporum* f. sp. phaseoli, Fos = *F. oxysporum* from soil, Fog = *F. oxysporum* from *Glycine max*, Fol = *F. oxysporum* f. sp. lycopersici, Fot = *F. oxysporum* f. sp. tracheiphilum, Foc = *F. oxysporum* from *Coffea arabica*, and Fov = *F. oxysporum* f. sp. vasinfectum.

trays were arranged on a greenhouse bench and seedlings were watered two to three times per day. Greenhouse air temperature was maintained at $23 \pm 5^\circ\text{C}$. Natural light was supplemented with high-pressure sodium grow lights (400 W) set for a photoperiod of light at 14 h day^{-1} . Two-week-old seedlings were washed under running tap water, with care taken not to damage roots. The roots were cut with scissors that were sterilized in 10% commercial bleach, leaving approximately 5 cm of root length. The remaining roots were then soaked in 30 ml of prepared inoculum for 10 min. To prepare the inoculum, isolates were grown on PDA plates for 5 days in an incubator set at 25°C . The conidia were dislodged with a pipette tip in 10 ml of sterile water and counted using a hemacytometer. The volume was adjusted by the addition of sterile water to obtain 1×10^6 conidia/ μl . Each inoculated plant was transplanted into steamed metro potting mix (Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada) in a 237-ml Styrofoam cup. Five plants were inoculated with each isolate. Noninoculated control plants with roots soaked in sterile water were included in the assay. The cups were grouped by isolate into five blocks. Foliar symptoms were scored weekly, starting 2 weeks after transplanting, using an ordinal scale from 1 to 5, where 1 = no symptoms and 5 = severe interveinal chlorosis and necrosis or dead plants (Hartman et al. 1997; Huang and Hartman 1998). The experiment was terminated after 6 weeks. Each plant was carefully removed from the cup and the roots were washed under running tap water. The plants were wrapped in a moist paper towel. Data for fresh root weight were collected. The stems and roots (hypocotyl area) were split longitudinally with an x-acto knife to examine for symptoms of vascular discoloration. Absence or presence of vascular discoloration was recorded for both the roots and stems. The pieces that were symptomatic and the noninoculated controls were cultured on PDA to recover *F. oxysporum*, if present. Briefly, the pieces of stems and roots were rinsed for 3 min in 1% NaHClO and 3 min for three times in sterile water. The tissue was blotted on a clean paper towel and placed on the culture medium. The assay for pathogenicity was conducted twice.

Correlations between foliar symptoms (3 weeks after transplanting) and vascular discoloration for stems, roots, and stems and roots combined were estimated using the Kendall τ_b correlation coefficient with PROC CORR of SAS 9.3 (SAS Institute, Inc., Cary, NC). Isolate aggressiveness was categorized based on the incidence of vascular discoloration (root and stem symptoms combined) from 10 plants, where high equals $\geq 70\%$ plants with symptoms, moderate equals $\geq 30\%$ plants with symptom incidence $< 70\%$, low equals $\geq 1\%$ plants with symptom incidence $< 30\%$, or nonpathogenic equals no plants with symptoms.

RNA isolation and real-time reverse-transcription PCR expression analysis. Fungal mycelium of the 13 *F. oxysporum* isolates having the *Six6* gene was ground in liquid nitrogen with a mortar and pestle, and total RNA was extracted from 1 g of sample using the TRIzol protocol (Invitrogen, Carlsbad, CA). RNA was purified with the RNA Clean-up protocol (Qiagen, Valencia, CA), according to the manufacturer's instructions, including the optional on-column DNase digestion, and stored at -80°C . The amount and the quality of the total RNA were estimated by measuring the concentration and A260/A280 and A260/A230 ratios with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), as well as by agarose gel electrophoresis. A 1- μg sample of total RNA was used for cDNA synthesis following the iScript cDNA synthesis kit protocol (Bio-Rad). Transcript abundances were identified using a reaction mix containing 12.5 μl of 2 $\times$ SYBR Green Mastermix (Applied Biosystems), 0.4 μM each primer, and 20 ng of single-strand cDNA template, adding RNase free water to reach the final volume of 25 μl . All analyses were performed on the CFX-96 device (Bio-Rad), according to the following conditions: 95°C for 3 min and 44 cycles at 95°C 10 s and 58°C 25 s. A melting curve analysis, ranging from 60 to 95°C , was used to identify different amplicons, including nonspecific products. Each sample was analyzed in triplicate together with a template-free negative control. *Six6* gene-specific primers were designed within consecutive exons, separated by an intron, using Primer3 software, and their sequences are here reported: Six6F (GGCTGAGCACAC

Fig. 2. Maximum parsimony phylogenetic tree of *Fusarium oxysporum* isolates from soybean and common bean inferred from the secreted in xylem 6 (*Six6*) gene sequence data. All sequences are followed by their GenBank accessions. Bootstrap values for the maximum parsimony and maximum-likelihood analyses (1,000 replications) and Bayesian posterior probabilities are indicated as percentages in that order at the internodes. *Colletotrichum fioriniae* strain PJ7 was used to root the tree. Fop = *F. oxysporum* f. sp. *phaseoli*, Fol = *F. oxysporum* f. sp. *lycopersici*, Fos = *F. oxysporum* from soil, and Fog = *F. oxysporum* from *Glycine max*.

CATCAATT) and Six6R (CTGTACACAAATCTCCCGCG). Relative quantification was normalized to the housekeeping control gene *tef1 α* (*tef1 α* F: GTCACCTTGATCTACCAAGTGCG; *tef1 α* R: GGTGACATAGTAGCGAGGAGT) and fold change (FC) in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008). This method allows for calculation of the expression ratio of a target gene between a test sample and its relative calibrator (Real-Time PCR Application Guide; Bio-Rad) which, in the present case, corresponded to the different FOSC isolates (test) compared with the isolate *F. oxysporum* f. sp. *phaseoli* 9455 (calibrator).

RESULTS

Sequence analysis and phylogeny. Overall, this analysis was carried out on sequences from 47 isolates: 22 of *F. oxysporum* f. sp. *phaseoli*, 17 of *F. oxysporum*, 5 of *F. commune*, 1 of *F. oxysporum* f. sp. *lycopersici* (NRRL34936) collected from tomato, 1 of *F. oxysporum* f. sp. *tracheiphilum* (ATCC16608) collected from cowpea, and 1 of *F. oxysporum* f. sp. *vasinfectum* (CML1098) collected from cotton (Table 1). The outcome of the *tef1 α* , mtSSU, and combined data analyses resulted in a total DNA alignment of 577, 615, and 1,171 positions, while 517, 593, and 1,113 bp were constant and used in the final analysis. Maximum parsimony analysis of *tef1 α* and mtSSU resulted in 9 and 10 most parsimonious trees, respectively, where consistency index (CI) = 0.872 and 0.920, retention index (RI) = 0.970 and 0.990, and composite index (COI) = 0.894 and 0.933, respectively. The strict consensus trees for maximum parsimony for *tef1 α* and mtSSU are shown in Supplementary Figures S1 and S2. The maximum-likelihood and Bayesian inference gave similar tree topologies for *tef1 α* and mtSSU when compared with the maximum parsimony analysis. The bootstrap values for the maximum-likelihood analyses and Bayesian posterior probabilities for the Bayesian analyses for *tef1 α* and mtSSU are reported. As previously described by Ellis et al. (2014), clades 2 and 3 were not clearly resolved in the trees generated from the *tef1 α* gene, and the *F. oxysporum* f. sp. *lycopersici*

isolate NRRL34936 fell between clade 2 and 3, whereas it grouped with the isolates of clade 2 for the mtSSU.

Maximum parsimony analysis of the combined dataset gave 10 most parsimonious trees (CI = 0.860, RI = 0.973, COI = 0.884). The strict consensus tree for maximum parsimony for the combined dataset along with the bootstrap values for the maximum-likelihood analysis and Bayesian posterior probabilities for the Bayesian analysis are reported in Figure 1. No major topological variations were observed among trees derived from maximum parsimony, maximum-likelihood (Supplementary Fig. S3), and Bayesian inference analysis (Supplementary Fig. S4) for the combined data, as well as for *tef1 α* and mtSSU. *F. oxysporum* isolates fell into clades 2, 3, and 5 and *F. commune* isolates fell into clade 6, as previously reported by Ellis et al. (2014). *F. oxysporum* f. sp. *phaseoli* isolates were distributed between clade 2 (41%) and clade 3 (59%). Clade 2 also included *F. oxysporum* f. sp. *tracheiphilum* (ATCC16608) and *F. oxysporum* f. sp. *vasinfectum* (CML1098), whereas *F. oxysporum* f. sp. *lycopersici* grouped in clade 3 (Fig. 1).

In summary, the phylogenetic analysis of *tef1 α* and mtSSU dataset revealed that all FOSC isolates tested in this study were topologically polyphyletic in origin, confirming the previous finding by Ellis et al. (2014).

The presence of the *Six6* gene was tested by PCR assays in all isolates listed in Table 1. A *Six6* amplicon was found to be present in 11 *F. oxysporum* f. sp. *phaseoli* isolates, the *F. oxysporum* f. sp. *lycopersici* isolate NRRL34936, and the *F. oxysporum* isolate ATCC62913. Sequences of *Six6* were determined from all isolates with the gene present. The DNA alignment resulted in 476 positions, while 468 bp were constant and used in the final analysis. The maximum parsimony analysis resulted in 10 most parsimonious trees (CI = 0.791, RI = 0.823, COI = 0.770). The strict consensus tree for maximum parsimony for *Six6* along with the bootstrap values for the maximum-likelihood analysis and Bayesian posterior probabilities for the Bayesian analysis are reported in Figure 2. Interestingly, high bootstrap values and Bayesian posterior probabilities support

Fig. 3. Disease phenotypes of soybean genotype MN1805: column 1, foliar symptoms; column 2, root discoloration; and column 3, stem lesions in response to Fusarium wilt. **A**, Mock-inoculated; **B**, ATCC62913-inoculated; and **C**, FOP8-inoculated.

the monophyly of *F. oxysporum* f. sp. *phaseoli* and *lycopersici* isolates. Sequencing of *Six6* amplicons revealed that they were identical within *F. oxysporum* f. sp. *phaseoli* and *lycopersici* but, notably, a divergence of *F. oxysporum* ATCC62913 was observed in *Six6* gene phylogeny. The gene was not present in isolates belonging to clades 4, 5, and 6 but the isolates in clades 2 and 3 were not clearly resolved by clade for the *Six6* gene (Table 1; Fig. 2). The amplification of the *Six6* gene in *F. oxysporum* f. sp. *lycopersici* NRRL34936, *F. oxysporum* f. sp. *phaseoli* 58, and *F. oxysporum* ATCC62913 isolates confirmed results previously obtained by Ellis et al. (2016). The three isolates are known to cause wilt to their respective hosts and also soybean (Ellis et al. 2016), thus suggesting a possible role of the *Six6* gene with pathogenicity toward soybean.

Fusarium wilt assay. In order to assess the link between the presence of the *Six6* effector gene and wilt capability of *F. oxysporum* isolates, inoculations using the susceptible soybean cultivar MN1805 were conducted with all *F. oxysporum* isolates used in this study. Foliar symptoms were best observed at 2 and 3 weeks after inoculation. The frequency of plants with symptoms and range of severity were the same for 2 and 3 weeks and, thus, are reported together in Table 1. Of the 47 isolates, 34 were able to infect soybean seedlings and cause foliar symptoms that included plants with symptoms ranging from leaves showing slight yellowing or chlorotic flecks or blotches to intervenal chlorosis and necrosis, or entirely defoliated plants. Control plants did not develop any symptoms (Fig. 3A). Notably, of the 34 isolates causing foliar symptoms, 28 also induced vascular discoloration (Table 1). Conversely, vascular discoloration in the stem and root but not foliar symptoms were observed in seedlings inoculated with two isolates: *F. oxysporum* f. sp. *phaseoli* FOPS026 and *F. oxysporum* f. sp. *lycopersici* NRRL34936. Both isolates amplified for the *Six6* gene. Foliar symptoms correlated moderately to strongly with vascular discoloration, depending on the timing of foliar symptom assessment; 3-week foliar symptoms had the best correlation with vascular discoloration. The correlations between combined root and stem vascular discoloration and 2- or 3-week foliar symptoms were 0.44638 ($P < 0.0001$) and 0.71064 ($P < 0.0001$), respectively. Aggressiveness was categorized as high, moderate, or low based on stem and root vascular discoloration comparisons with known check isolates. In all, 9 isolates were ranked as high, 12 as moderate, and 7 as low, while the remaining isolates did not cause wilt symptoms on soybean (Table 1). The isolates with the highest incidence of vascular discoloration (10 of 10 plants with stem and

root lesions) and severe foliar symptoms (10 plants with a score ranging from 2 to 5) included FOP31, FOP52, FOP58, FOP8, and *F. oxysporum* ATCC62913. FOP31, FOP58, and FOP8 were previously reported as highly virulent vascular wilt pathogens in common bean (de Sousa et al. 2015). Foliar symptoms and vascular discoloration in the root and stem elicited by *F. oxysporum* ATCC62913 and FOP8 in soybean genotype MN1805 can be seen in Figure 3B and C, respectively.

Most wilt-inducing isolates grouped in clade 3 (approximately 43%), followed by clades 2, 6, and 5, which included 32, 18, and 7% of isolates, respectively, producing wilt symptoms on soybean (Table 1). Although the *Six6* gene was not present in all isolates causing wilt symptoms on soybean, all isolates with a copy of the *Six6* amplicon produced wilt symptoms on soybean, and two-thirds of these isolates were defined as highly aggressive. These results suggest a positive association between the presence of the effector gene *Six6* and capability to cause wilt symptoms in soybean.

Expression analysis of the *Six6* gene. The expression profile of the effector gene *Six6* was analyzed by real-time reverse-transcription (RT)-PCR. In order to explore the differences among the FOSC isolates that had a copy of the *Six6* gene, FC were measured by comparing all positive isolates with the *F. oxysporum* f. sp. *phaseoli* isolate 9455 (Fig. 4).

The upregulation of *Six6* occurred in all tested isolates, with values ranging from 1,943.3 to 4.4, with the highest and lowest values for isolates *F. oxysporum* ATCC62913 (highly aggressive) and FOPS019 (moderately aggressive), respectively. The maximal levels of expression were reached by the isolates that caused more severe wilt symptoms in the greenhouse assay. In addition to the *F. oxysporum* ATCC62913 isolate, enhanced induction also was observed in *F. oxysporum* f. sp. *phaseoli* isolates ATCC18131, FOP31, FOP58, FOP8, and FOP52. The *F. oxysporum* f. sp. *lycopersici* isolate NRRL34936, although moderately aggressive as a wilt isolate, showed strong induction up to 50-fold; whereas, in contrast, weaker alterations were measured in the remaining moderately aggressive wilt isolates (Fig. 4).

DISCUSSION

In this study, genotypic and phenotypic diversity within the FOSC isolates was reported. *F. oxysporum* f. sp. *phaseoli* isolates collected from common bean and additional *F. oxysporum* isolates collected from soybean were polyphyletic based on *tef1α* and mtSSU phylogeny. These findings confirmed the phylogenetic analysis previously described by Ellis et al. (2014), revealing that isolates within FOSC from soybean were polyphyletic in origin, with soybean isolates belonging to five clades within the FOSC, including *F. commune*. Common bean isolates were distributed among clades 2 and 3. The phylogenetic divide indicated that formae speciales of *F. oxysporum*, including *F. oxysporum* f. sp. *phaseoli*, had multiple evolutionary origins, as already stated in other studies using different FOSC in other hosts such as banana (O'Donnell et al. 1998), common bean (Alves-Santos et al. 1999), bulb species (Baayen et al. 2000), cotton (Abo et al. 2005), soil (Laurence et al. 2012), date palm (Laurence et al. 2015), and soybean (Ellis et al. 2014).

Despite being polyphyletic based on *tef1α* and mtSSU loci, the putative effector gene *Six6* was found to be monophyletic for *F. oxysporum* f. sp. *phaseoli* and *F. oxysporum* f. sp. *lycopersici* isolates belonging to clades 2 and 3 within the FOSC. Nucleotide sequences of the *Six6* gene for all *F. oxysporum* f. sp. *phaseoli* isolates containing a copy of the gene and the *F. oxysporum* f. sp. *lycopersici* isolate NRRL34936 collected from tomato were identical. *F. oxysporum* isolate ATCC62913 collected from soybean had a unique nucleotide sequence, with notable divergence in *Six6* gene phylogeny. Therefore, for all isolates, with the exception of *F. oxysporum* ATCC62913, this would suggest that the effector gene

Fig. 4. Expression of *Six6* gene assayed by real-time reverse-transcription polymerase chain reaction from different isolates in the *Fusarium oxysporum* species complex. Relative quantification of *Six6* gene cycle threshold (Ct) values was normalized to the housekeeping translation elongation factor (*tef1α*) gene. Values represent the number of times *Six6* is expressed by the different *F. oxysporum* species complex isolates compared with the isolate *F. oxysporum* f. sp. *phaseoli* 9455. H, M, and L indicate high, medium, and low levels of aggressiveness, respectively, as shown by greenhouse assays.

Six6 has a single origin and that horizontal gene transfer (HGT) explains the presences and absences of the gene within and across clades 2 and 3 of the FOSC (Baayen et al. 2000; Fourie et al. 2011; O'Donnell et al. 1998). With the rapid advances in sequencing technologies, there has been a significant increase in genomic evidence to support the HGT hypothesis of pathogenicity factors in *F. oxysporum*. Multiple studies reported that the nucleic acid and amino acid sequences of *Six* genes are highly conserved both within and between formae speciales (Houterman et al. 2008; Rep et al. 2004; Thatcher et al. 2012; Van Der Does et al. 2008). This high degree of sequence conservation is thought to indicate the recent evolutionary origin of *Six* genes that may have then been horizontally transferred between strains. Ma et al. (2010) investigated lineage-specific regions in *F. oxysporum* f. sp. *lycopersici* and whether these regions were acquired by horizontal transfer. The lineage-specific regions in *F. oxysporum* f. sp. *lycopersici* are enriched for genes related to host-pathogen interactions, including chromosome 14, which is where the *Six* genes are mostly located (Lievens et al. 2009; Ma et al. 2010; Rep and Kistler 2010). Ma et al. (2010) concluded that the mobility of lineage-specific chromosome 14 between strains explains its presence in tomato wilt pathogens, comprising several clonal lineages polyphyletic within the FOSC, but absence in other lineages (Van Der Does et al. 2008). Furthermore, Ma et al. (2010) also determined that horizontal transfer of host-specificity factors between *F. oxysporum* species may explain the polyphyletic origins of *F. oxysporum* as well as their host specialization.

In order to determine possible correlations between the presence of the *Six6* effector gene and *F. oxysporum* pathogenicity, the ability to cause wilt symptoms in soybean was assayed in this study. Previously, the *F. oxysporum* isolates collected from soybean were tested in a greenhouse study to determine their ability to produce vascular wilt (Ellis et al. 2016). The three isolates producing high levels of vascular wilt on soybean (ATCC62913, FO93, and FOP58) were also confirmed to be highly aggressive in the current study. In addition, all *F. oxysporum* f. sp. *phaseoli* isolates that produced symptoms of wilt on soybean were also pathogenic toward common bean, according to de Sousa et al. (2015). In our study, seven *F. oxysporum* f. sp. *phaseoli* isolates (2256-1, FOPS018, LAPS152, LAPS153, LAPS154, LAPS155, and LAPS156) did not cause wilt symptoms in soybean. The same seven *F. oxysporum* f. sp. *phaseoli* isolates are highly virulent wilt pathogens to common bean (de Sousa et al. 2015). Interestingly, these isolates did not amplify for the *Six6* gene but did amplify for the *ftf1* transcription factor (de Sousa et al. 2015). Factor *ftf1* was previously determined to be highly expressed during infection of common bean by *F. oxysporum* f. sp. *phaseoli* in highly aggressive isolates but not in weakly aggressive and nonpathogenic isolates (Ramos et al. 2007). Transcription factor *ftf1* is included in a family of related genes in *F. oxysporum* f. sp. *lycopersici*, with 3 of the 11 members occurring on pathogenicity chromosome 14 (Ma et al. 2010). The work from Chakrabarti et al. (2011) suggested that a homolog of the *ftf1* transcription factor, identified from common bean isolates, might be linked with the *Six6* effector from populations of *F. oxysporum* cotton isolates in Australia. In the present study, we could not determine a real association between the two genes because, in some cases, *F. oxysporum* f. sp. *phaseoli* isolates that carried *ftf1* gene (de Sousa et al. 2015) did not amplify for the *Six6* gene. Furthermore, although all isolates with a copy of *Six6* amplicon produced wilt symptoms on soybean, some isolates lacking *Six6* were moderately virulent and others with the gene were only weakly virulent, making further analysis of this gene necessary to clarify its diagnostic value.

In our study, the presence of the *Six6* gene was also associated with high levels of gene expression, in particular in the *F. oxysporum* isolates that caused more severe wilt symptoms, as shown by real-time RT-PCR. Previously, the gene expression patterns of several virulence factors were analyzed and compared in the interaction of two *F. oxysporum* f. sp. *phaseoli* strains displaying clear differences in virulence with a susceptible common bean

cultivar (Niño-Sánchez et al. 2015). Interestingly, the genes encoding *ftf1*, *Six1*, and *Six6* were only upregulated at an enhanced level in the highly virulent strain in root, crown, and hypocotyl tissue, matching with the beginning of massive colonization observed for this strain (Niño-Sánchez et al. 2015). Further studies on the expression of *Six6* gene in planta during infection and gene knock-out experiments may be required to help elucidate its role in pathogenicity.

Our results suggest the suitability of the *Six6* gene as a possible locus for pathogenicity-based molecular diagnostics across the various formae speciales, indicating that this gene may be dispersed more broadly across the FOSC as a whole. Nevertheless, future work should include experiments aimed at functional characterization of the *Six6* gene within *F. oxysporum* isolates and identification of the target site and interactions with affected hosts to elucidate how this effector contributes to virulence of *F. oxysporum* isolates for hosts such as soybean and common bean.

ACKNOWLEDGMENTS

This project was funded by the Iowa Soybean Association. We thank D. Cruz and D. Mayfield for technical assistance and cooperation in this study and A. Kriss for her assistance with the statistical analysis.

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